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ORIGINAL ARTICLE

Rapid and highly specific screening for *NPM1* mutations in acute myeloid leukemia

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Abstract *NPM1* mutations, the most frequent molecular alterations in acute myeloid leukemia (AML), have become important for risk stratification and treatment decisions for patients with normal karyotype AML. Rapid screening for *NPM1* mutations should be available shortly after diagnosis. Several methods for detecting *NPM1* mutations have been described, most of which are technically challenging and require additional laboratory equipment. We developed and validated an assay that allows specific, rapid, and simple screening for NPM1 mutations. FAST PCR spanning exons 8 to 12 of the *NPM1* gene was performed on 284 diagnostic AML samples. PCR products were visualized on a 2 % agarose E-gel and verified by direct sequencing. The FAST PCR screening method showed a specificity and sensitivity of 100 %, i.e., all mutated cases were detected, and none of negative cases carried mutations. The limit of detection was at 5–10 % of mutant alleles. We conclude that the FAST PCR assay is a highly specific, rapid (less than 2 h), and sensitive screening method for the detection of *NPM1* mutations. Moreover, this method is inexpensive and can easily be integrated in the routine molecular diagnostic work-up of established risk factors in AML using standard laboratory equipment.

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Introduction

In acute myeloid leukemia (AML), an increasing number of genetic alterations have been described to be involved in the pathogenesis of the disease, and to determine the phenotype and the impact of the treatment [1]. As a consequence, the identification of molecular alterations by cytogenetic and/or molecular analysis is of great importance for risk stratification and treatment decisions. Molecular analyses are particularly important in AML patients with a normal karyotype who constitute about half of all AML cases [2].

NPM1 mutations represent the most frequent alterations in AML, occurring in approximately one third of adult patients with AML, and are associated with a normal karyotype in 85 % of cases [3]. In the 2008 World Health Organization (WHO) classification, AML with mutated nucleophosmin (NPM1) has been defined as a separate provisional entity, and there is increasing evidence that *NPM1* mutations represent founder genetic events in AML. Various studies have demonstrated the favorable prognostic impact of NPM1 mutations especially in the absence of a *FLT3* ITD [4–8].

On the molecular level, *NPM1* mutations are insertions that cluster within exon 11 and 12 of the *NPM1* gene. The insertions typically consist of 1 or 2 tetranucleotides at nucleotides 956 to 971 (reference sequence NM_002520) corresponding to nucleotides c.860 through c.875 (HGVS nomenclature) [9]. The most prevalent mutation is the 960insTCTG (type A mutation) that is present in approximately 80 % of cases. Rare cases of non-tetrameric insertions and deletions/insertions have been described as well as single cases of 8-bp insertions in exon 11 leading to a truncated protein [10, 11]. All mutations in exon 12 lead to a frame-shift and an elongated protein

with loss of the nucleolar localization domain and/or gain of a nuclear export signal resulting in an accumulation of the protein in the cytoplasm. A variety of molecular methods for detecting *NPM1* mutations have been described, including PCR amplification and direct sequencing, high-resolution fragment analysis based on a fluorescence-labeled forward primer (Genescan), melting curve analysis on the LightCycler system, denaturing high-performance liquid chromatography (DHPLC), and LNA-mediated PCR clamping [12]. These methods are technically challenging and require well-trained personnel and additional laboratory equipment that might not be available in many molecular routine laboratories. Hence, since the detection of *NPM1* mutations at diagnosis is important for risk stratification and treatment decision, molecular laboratories should be able to perform the initial analysis using their standard laboratory equipment. We therefore developed a rapid and simple screening assay based on FAST PCR amplification and standardized agarose gel electrophoresis.

Materials and methods

Patient samples

We validated our assay on 284 diagnostic samples from adult patients with AML referred to our laboratory, including 158 *NPM1*-mutated (*NPM1*-mt) samples and a series of 126 in-house *NPM1*-wild-type (*NPM1*-wt) samples. Patients were diagnosed according to the WHO criteria at the University Hospitals of Bern, Basel, and Zürich and the District Hospitals of Lucerne, Bellinzona, St. Gallen, Thun, and Aarau. All patients were enrolled in the multicenter prospective trials of the HOVON/SAKK Study Group (HOVON AML protocols 42, 43, 81, 92, 102, 103) or treated according to the HOVON/SAKK protocols after informed consent according to the declaration of Helsinki. Patient characteristics are given in Table 1. All samples of bone marrow or peripheral blood were collected at diagnosis.

Table 1 Patient characteristics

	NPM1 mutated (n=158)		*NPM1* wild-type (n=126)		p value
	no.		no.		
Age at diagnosis, years, median (range)		58 (17–87)		56 (20–78)	0.116
Sex F/M		86/72		49/77	0.009
WBC, $\times 10^9$/L, median (range)	145	26.3 (0.2–346.6)	126	6.6 (0.7–183.9)	< 0.0005
% Blasts in blood, median (range)	132	40.5 (0–98)	103	39.5 (0.5–95)	0.807
FAB classification	135		122		
M0		3 (2.2)		24 (19.7)	< 0.0005
M1		30 (22.2)		28 (23.0)	0.889
M2		39 (28.9)		29 (23.8)	0.353
M4		25 (18.5)		19 (15.6)	0.531
M5		34 (25.2)		4 (3.3)	< 0.0005
M6		1 (0.7)		6 (4.9)	0.056
M7		0		3 (2.6)	0.106
RAEB-2		3 (2.2)		7 (5.7)	0.200
Secondary AML following MDS		0		2 (1.6)	0.224
Cytogenetics	131		126		
Normal karyotype, no. (%)		106 (80.9)		61 (48.4)	< 0.0005
Favorable[a], no. (%)		0		10 (7.9)	0.001
Intermediate-II[a], no. (%)		18 (13.7)		14 (11.1)	0.523
Adverse[a], no. (%)		7 (5.3)		41 (32.5)	0.001
Molecular genetics					
AML1-ETO, no. (%)	150	0	118	7 (5.9)	0.003
CBFb-MYH11, no. (%)	149	0	118	3 (2.5)	0.085
CEBPA mutations, no. (%)	23	2 (8.7)	43	5 (11.6)	0.655
FLT3 ITD, no. (%)	147	62 (42.2)	121	6 (5.0)	< 0.0005
EVI1 overexpression, no. (%)	14	0	21	1 (4.4)	1.00

[a] Cytogenetic groups according to ELN classification (13). Favorable: t(8;21)(q22;q22); inv(16)(p13.1;q22) or t(16;16)(p13.1;q22). Intermediate-II: t(9;11)(p22;q23); cytogenetic abnormalities not classified as favorable or adverse. Adverse: inv(3)(q21;q26.2) or t(3;3)(q21;q26.2); t(6;9)(p23;q34); t(v;11)(v;q23); -5 or del(5q); -7; abnl(17p); complex karyotype

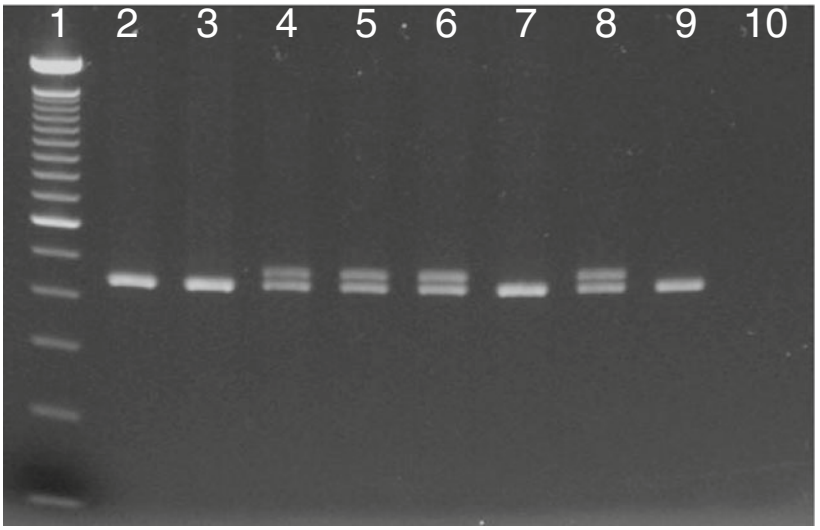

Fig. 1 After FAST PCR, PCR products are visualized on a 2 % agarose E-gel, where a *double band* indicates a mutation and a *single band* represents the wildtype sequence. *Lanes 1* 100 bp ladder, *2*, *3* and *7* patient samples with wild-type NPM1, *4–6* patient samples with mutated NPM1, *8* positive control cell line OCI-AML3, *9* wild-type control cell line HL60, *10* no template control

Mutational analysis

Total RNA was extracted from Ficoll-separated mononuclear cells using the QIAmp RNA Blood Mini Kit (Qiagen, Hombrechtikon, Switzerland) and reverse transcribed by the Superscript II system (Invitrogen, Lucerne, Switzerland). For mutational analysis, a primer pair was designed to amplify exons 8 to 12 of the *NPM1* gene (NPM663F 5′-GCGCCAGTGAAGAAATCTATAC-3′ and NPM1109R 5′-GGACAACATTTATCAAACACGG-3′) yielding a 446-bp product. Two microliters of cDNA were amplified in a volume of 20 μl containing 0.5 μM of each primer and 10 μl of 2× GeneAmp Fast PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland) on a 9800 or Veriti Fast Thermal Cycler (Applied Biosystems). Reaction conditions consisted of an initial denaturation step at 94 °C for 10 s followed by 35 cycles at 94 °C for 0 s, 60 °C for 5 s, and 72 °C for 20 s and a final elongation step at 72 °C for 30 s. PCR products were run for 40 min on a 2 % agarose E-gel (Invitrogen) where a double band indicates a mutation and a single band represents the wild-type sequence. All PCR products were verified by direct sequencing in both directions using BigDye Terminator Mix version 3 (Applied Biosystems, Rotkreuz, Switzerland) on an ABI3130xl automated sequencer. PCR products yielding a small smear above the wild-type band were sequenced with primers NPM793F (5′-AAAACTCCTAAAACACCAAAAGGACC-3′), NPM883F (5′-AAAGTGGAAGCCAAATTCATCAA-3′), NPM845R (5′-CACCTTTTTCTATACTTGTTGCATTT-3′), and NPM964R (5′-TATTTTCTTAAAGAGACTTCCTCCA-3′).

With FAST PCR, thermal cycling reactions that normally take over an hour and a half can take as little as 25 min. The FAST PCR technique is performed on designated FAST PCR thermal cyclers and optimized reagents must be used. Alternatively, our PCR protocol was also run by standard conditions on an ABI 9700 Thermal Cycler (Applied Biosystems) using Roche Taq polymerase (Roche, Rotkreuz, Switzerland) and buffer. Reaction conditions consisted of denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s and a final elongation at 72 °C for 5 min. Results were consistent, but PCR products were somewhat fainter probably due to a lower PCR efficiency.

Statistical analysis

Statistical analyses were performed on SPSS 17.0 for Windows (SPSS Inc., Chicago, Illinois). The nonparametric Wilcoxon–Mann–Whitney test was used for continuous data and the χ^2 or Fisher exact (whenever an expected value was <5) test for categorical data to compare demographic, laboratory and molecular parameters between the patient groups. A p value of <0.05 was considered statistically significant.

Results

Of 284 AML samples included in the study, 158 were *NPM1* mutated and 126 were wild-type as determined by FAST PCR screening. Mutated PCR products invariably showed two distinct bands on agarose gel electrophoresis, probably based on heteroduplex formation (Fig. 1). All mutated and wild-type PCR products were directly sequenced in both directions, and the results were 100 % concordant with the screening method. Mutations included 151 typical 4-bp insertions and 6 uncommon mutations in exon 12 (Table 2). In addition, a 19-bp deletion was detected in exon 9 outside the commonly mutated region leading to a frame-shift and a premature stop codon.

In 2 samples with mutated and 10 samples with wild-type *NPM1*, a novel 3-bp in frame-deletion in exon 8 (748delTCA) was identified resulting in a loss of serin 218 (*p.Ser218del*). We detected this sequence variation in samples of patients with other hematological malignancies and healthy donors at variable proportions (CML, 0/18; ALL, 3/18; CLL, 5/12; healthy donors, 14/15) suggesting to represent a common polymorphism. The frequency and potential relevance of this sequence variation is currently under investigation in our laboratory.

Specificity and sensitivity of FAST PCR screening

The specificity and diagnostic sensitivity of the FAST PCR screening method were 100 %, i.e., all positive samples carried mutations and none of the 126 negative cases were mutated (positive predictive value of 1.00 and negative predictive value of 1.00). The limit of detection (LOD) of the assay as assessed by serial dilution experiments of the cell line OCI-AML3 in the negative cell line HL60 was 5–10 % mutated alleles.

NPM1 mutations and biological and morphological features

The incidence of *NPM1* mutations was higher in women (*NPM1*-mt, women, n=86 (54 %); men n=72; *NPM1*-wt, women, n=49 (39 %), men n=77, p=0.009). The median ages of patients with and without *NPM1* mutations were 58 and 56 years, respectively. Patients with *NPM1* mutations had significantly higher median white blood cell counts than patients without *NPM1* mutations (26.3×10^9/L versus 6.6×10^9/L; $p<0.0005$). No difference was observed with regard to the peripheral blast count (*NPM1*-mt, median, 40.5; *NPM1*-wt, median, 39.5). *NPM1* mutations were predominantly found in the French–American–British (FAB) subgroups M1, M2, M4, and M5, and were less frequent in M0, M6, and M7. When compared to the *NPM1*-wt group, *NPM1* mutations were significantly less frequent in FAB M0 and more frequent in FAB M5 (FAB M0, 2.2 % versus 19.7 %; $p<0.0005$; FAB M5, 25.2 % versus 3.3 %; $p<0.0005$).These data confirm the known features of many reports reviewed in [3].

NPM1 mutations and cytogenetic and molecular features

Cytogenetic data were available for 257 of the 284 patients with AML. In accordance with results from other studies, *NPM1* mutations were significantly associated with a normal karyotype (*NPM1*-mt, 80.9 %; *NPM1*-wt, 48.4 %; $p<0.0005$) [3]. Patients with an aberrant karyotype were classified in cytogenetic risk groups according to the recommendations of the European LeukemiaNet [13]. We found significantly less patients with *NPM1* mutations in the favorable and adverse cytogenetic subgroups compared to patients without *NPM1* mutations (favorable, 0 versus 7.9 %; p=0.001; adverse, 5.3 % versus 32.5 %; p=0.001); 13.7 % and 11.1 % of patients with and without *NPM1* mutations carried chromosomal aberrations of type

Table 2 NPM1 mutations detected by FAST PCR screening

Type of mutation	Exon	Nucleotide sequence	Protein	Number	% of mutated
Wild-type	12	CAAGATC----TCTG--------GCAG----T----GGAGGAAGTCTCTTT	MTDQEAIQDL**W**Q**W**RKSL	126	
A	12	CAAGATC----TCTG***TCTG***----GCAG----T----GGAGGAAGTCTCTTT	MTDQEAIQDLCLAVEE**VSLRK**	117	75
B	12	CAAGATC----TCTG***CATG***----GCAG----T----GGAGGAAGTCTCTTT	MTDQEAIQDLCMAVEE**VSLRK**	16	10
D	12	CAAGATC----TCTG***CCTG***----GCAG----T----GGAGGAAGTCTCTTT	MTDQEAIQDLCLAVEE**VSLRK**	9	6
G	12	CAAGATC----TCTG***TTTG***----GCAG----T----GGAGGAAGTCTCTTT	MTDQEAIQDLCLAVEE**VSLRK**	3	2
IV/DD3	12	CAAGATC----TCTG***CAGA***----GCAG----T----GGAGGAAGTCTCTTT	MTDQEAIQDLCRAVEE**VSLRK**	1	<1
DD5	12	CAAGATC----TCTG***TCAG***----GCAG----T----GGAGGAAGTCTCTTT	MTDQEAIQDLCQAVEE**VSLRK**	1	<1
D7	12	CAAGATC***ATGT***TCTG--------GCAG----T----GGAGGAAGTCTCTTT	MTDQEAIQDHVLAVEE**VSLRK**	1	<1
Gm	12	CAAGATC----TCTG***CAGG***----GCAG----T----GGAGGAAGTCTCTTT	MTDQEAIQDLCRAVEE**VSLRK**	1	<1
J	12	CAAGATC----TCTG***TATG***----GCAG----T----GGAGGAAGTCTCTTT	MTDQEAIQDLCMAVEE**VSLRK**	1	<1
Nm	12	CAAGATC----TCTG***CCAG***----GCAG----T----GGAGGAAGTCTCTTT	MTDQEAIQDLCQAVEE**VSLRK**	1	<1
960insYTTG	12	CAAGATC----TCTG***YTTG***----GCAG----T----GGAGGAAGTCTCTTT	MTDQEAIQDLCLAVEE**VSLRK**	1	<1
960insTATTGGG	12	CAAGATC----TCTG***TATTGGG***-GCAG----T----GGAGGAAGTCTCTTT	MTDQEAIQDL**W**Y**W**AVEE**VSLRK**	1	<1
964insAAAA	12	CAAGATC----TCTG--------GCAG**AAAA**T----GGAGGAAGTCTCTTT	MTDQEAIQDL**W**QKMEE**VSLRK**	1	<1
965-970delinsCCCTCTCTCG	12	CAAGATC----TCTG--------GCAG----T***CCCTCTCTCG***AGTCTCTTT	MTDQEAIQDL**W**QSLSR**VSLRK**	1	<1
966-970delinsTTTTAGTCC	12	CAAGATC----TCTG--------GCAG----TG***TTTTAGTCC***AGTCTCTTT	MTDQEAIQDL**W**QCFSP**VSLRK**	1	<1
933_936delTGAC 960insTCTGTCTG	12	CAAGATC----TCTG***TCTGTCTG***GCAG----T----GGAGGAAGTCTCTTT	MTKRLFKISVCLAVEE**VSLRK**	1	<1
849_867del	9	AGCAAAAATG----------GTGGTTCTCTTCCCAAA	KAKM**VVLFPKWKPNSSIM**stop	1	<1

Genbank RefSeq Accession number NM_002520

intermediate II. Analysis of concomitant molecular aberrations confirmed the significant known association of *NPM1* mutations and *FLT3* ITD (42.2 % versus 5 %; $p<0.0005$). No *AML1-ETO* and *CBFb-MYH11* fusions were found in the *NPM1*-mutated group. The frequencies of *CEBPA* mutations and *EVI1* overexpression were not different in patients with and without *NPM1* mutations.

Discussion

Given the favorable prognostic value of the *NPM1*+/*FLT3* ITD− genotype and the resulting therapeutic consequences, a rapid and simple method to assess the presence of *NPM1* mutations is required. In this report, we show that FAST PCR and subsequent gel electrophoresis is a specific and robust tool for the screening of *NPM1* mutations and can be performed in less than 2 h. PCR products are run on a ready-to-use 2 % agarose gel that clearly separates the mutant from the wild-type band. The remaining PCR product can directly be sequenced for accurate determination of the type of mutation. Although the prognostically favorable phenotype of *NPM1* mutations is independent of the type of mutation, it is important to identify the precise mutation for the design of real-time primers and probes for subsequent monitoring of minimal residual disease by qPCR.

In contrast to other methods, both the specificity and diagnostic sensitivity of the FAST PCR screening assay are 100 % (methods reviewed in [12]). The LOD of 5–10 % mutated alleles equals the sensitivity of other methods such as capillary electrophoresis and is higher than for direct sequencing. For minimal residual disease detection, more sensitive methods such as allele-specific qPCR are appropriate, yet at the price of a limited diagnostic sensitivity that is restricted to one specific mutation per assay.

The FAST PCR technique has the advantage of reducing time-to-results by approximately a half to two thirds. Many but not all standard PCR protocols can be converted with little effort. The protocol presented in this report can also be run with standard conditions but yields the most PCR product by the FAST PCR mode.

We conclude that FAST PCR followed by agarose gel electrophoresis allows highly specific, rapid and sensitive screening for *NPM1* mutations. More precisely, all mutations were detected and none was missed by this method. In addition, our assay is inexpensive and can easily be integrated in the routine molecular diagnostic work-up of established risk factors in AML using standard laboratory equipment.

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Conflict of interest The authors declare that they have no conflict of interest.

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